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ABSTRACT

Vaccination of neonates with Mycobacterium bovis bacillus Calmette-Guerin (BCG) may be a strategy that overcomes reduced vaccine efficacy associated with exposure to environmental mycobacteria in humans and cattle. Preliminary comparisons indicated that 2-week-old calves produced an immune response to vaccination at least as intense as that observed in adults. Subsequently, five gnotobiotic hysterotomy derived calves aged 1 day were inoculated with BCG and 3 months later were challenged intranasally with virulent M. bovis. The number of tissues with lesions and the pathological extent of these lesions was reduced significantly in vaccinates. Furthermore, lesions were evident in the lung or associated chest lymph nodes of four of five controls but none of five vaccinates. BCG vaccination reduced significantly the level of bacterial colonization. However, lesions in the head associated lymph nodes were observed in three of five BCG-vaccinated cattle. Levels of interferon gamma (IFN-gamma) detected by enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunospot (ELISPOT) in individual vaccinated animals at challenge did not correlate with subsequent resistance and in general immune responses post-challenge were lower in vaccinated calves. Low IL-10 responses were evident but IL-4 was not detected. Responses to ESAT-6 and/or CFP-10 were evident in four of four control calves that had lesions. Two of the BCG vaccinates with lesions did not produce a response to ESAT-6 and CFP-10, indicating that these antigens did not distinguish vaccinated immune animals from vaccinated animals with lesions. Overall, vaccination of neonatal calves with BCG induced significant protection against disease and has potential as a strategy for the reduction of the incidence of bovine tuberculosis.

Keywords: BCG, Mycobacterium bovis, interferon gamma, neonatal vaccination

Introduction

In many countries the persistence or increased incidence of bovine tuberculosis (TB) poses a human health risk and a major economic problem. Bacillus Calmette–Guérin (BCG) was developed as a human tuberculosis vaccine in the 1920s, but despite its widespread use has a variable efficacy against pulmonary TB, ranging from 0% to 80%[1,2]. Meta-analysis of human BCG trials revealed that BCG vaccination was most successful when administered during childhood and that this induced protection against meningeal and miliary TB more effectively than against pulmonary TB [2].

A number of hypotheses exist for the variable efficacy of BCG vaccination. One is interference or masking of protection by previous exposure to environmental mycobacteria [2]. Previous exposure may reduce the immunity induced by BCG vaccination, effective vaccine 'take' may be prevented [3], or previous exposure could prime an inappropriate bias in the immune response. On the other hand, there is evidence that exposure to environmental mycobacteria may give a low level of protection against *Mycobacterium tuberculosis* that cannot be improved upon by vaccination with BCG [4].

The highest prevalence of human TB is in Africa, Asia and Central America, where there is also a high incidence of exposure to environmental mycobacteria. Studies in Malawi indicated that pre-existing responses to mycobacterial antigens [purified protein derivative (PPD)] was associated with reduced interferon (IFN)-γ responses post-BCG vaccination in adults [5,6]. In contrast, vaccination of infants at birth in The Gambia was shown to induce strong IFN-γ responses in the absence of interleukin (IL)-4 [7,8]. These neonates had no pre-existing IFN-γ or IL-4 responses to PPD. However, infants studied at 4 months of age had considerable pre-existing

IL-4 responses to mycobacterial antigen and less IFN-γ secretion was observed post-vaccination. These data indicate the importance of early vaccination for strong Th1-biased immunity.

TB in cattle has a similar pathogenesis to that in humans and the kinetics of immune responses post-vaccination and post-virulent challenge can be measured easily [9]. Cattle, like humans and unlike mice, are immunocompetent at birth. In addition, cattle studies have also revealed variable efficacy of BCG vaccination [10–12]. Calves are usually sensitized to mycobacterial antigens early after birth and conventionally reared calves show responses to PPD by 6 weeks of age. Vaccination of adult cattle with pre-existing responses to environmental mycobacteria was much less effective than seen in animals without pre-existing responses [12].

Previously, we have demonstrated that BCG vaccination of 1-day-old gnotobiotic, germ-free calves provoked a strong PPD-B specific immune response [13,14]. Studies using conventionally reared neonatal cattle indicated that BCG vaccination within 8 h of birth can provide very efficient protection against intratracheal/endobronchial challenge with virulent *M. bovis*[15]. However, a major constraint to the use of BCG as a vaccine in cattle is that it interferes with detection of TB by current skin and IFN-γ tests, and diagnostic tests utilizing *M. bovis*-specific antigens are being developed [16–20].

In a preliminary experiment we compared the response of neonatal and adult cattle to vaccination with BCG. The neonates responded more effectively than the adults, as judged by IFN-γ production by lymphocytes cultured with PPD (unpublished observations). If reduced responses to BCG vaccination result from environmental exposure it is proposed that neonatal vaccination could avoid this by priming for an appropriate response. The focus of this study was to investigate the protective

efficacy of BCG vaccine when administered to neonatal calves. However, such studies provide valuable opportunities to test potential diagnostic antigens that may be useful in distinguishing vaccinated from infected cattle. This has been tested in a model in which calves were challenged intranasally, and the ability of the *M. bovis*-specific antigens ESAT-6 and CFP-10 assessed for ability to detect vaccinated but challenged and diseased animals.

MATERIALS AND METHODS

Bacteria

BCG Pasteur was diluted from previously titrated frozen (−70°C) stock grown in Middlebrook 7H9 broth containing 10% ADC supplement [21]. Numbers of colony-forming units (CFU) were determined on 7H10 agar plates. *M. bovis* strain AF 2122/97 [22] grown in 7H9 broth for 7 days was frozen at − 70°C. This strain was isolated in 1997 from a typical case of bovine TB in the United Kingdom and is a common spoligotype. It has been shown previously to be pathogenic to cattle by intratracheal instillation. A 1-ml aliquot was thawed and cultured in 10 ml of 7H9 broth for 7 days at 37°C to produce log phase cells. The OD600 was measured and the culture was diluted in 7H9 broth to give an estimated 5×10^3 CFU per ml based on a comparison with a previously established standard curve. The number of CFU was determined on 7H11 agar containing 10% OADC supplement and 4·16 g pyruvate per litre.

Animals and experimental plan

British Holstein–Friesian calves (*Bos taurus*) were produced from cows inseminated with semen from major histocompatibility complex (MHC) homozygous bulls. One bull was A18/A18, the other A31/A31. All animals were bred at the Institute for Animal Health [23].

Two groups of five gnotobiotic calves derived by hysterotomy into plastic isolators [24] were inoculated subcutaneously with 10^6 CFU BCG or 7H9 medium at 1 day of age. Calves were transferred from the isolator into a high security unit when approximately 4 weeks of age, as dictated by UK animal welfare regulations, challenged intranasally with $1{\cdot}3 \times 10^4$ virulent *M. bovis* when aged 12 weeks and necropsied 16 weeks later.

The experiments were approved by the local ethics committee according to national UK guidelines.

Post-mortem examination and bacteriology

Lymph nodes of the head (retropharyngeal, submandibular, parotid) and thorax (mediastinal and four bronchus-associated), tonsils, nasal and tracheal mucosa and the seven pulmonary lobes were examined for gross lesions following the cutting of 0·5–1 cm slices. Macroscopic lesions were scored as described previously [25]. Briefly, lymph nodes were assigned a score of 0 for no visible lesions, 1 for one to two small foci, 1–2 mm in diameter, 2 for several small foci 1–2 mm in diameter or a necrotic area 5 by 5 mm, 3 for multiple lesions or extensive necrotic areas and 4 for gross lesions extending over much of the lymph node. Lungs were assigned a value of 0 for no visible lesions, 1 for no gross lesions but lesions apparent on slicing, 2 for up

to 5 lesions <10 mm diameter, 3 for more than six lesions <10 mm in diameter, 4 for one distinct lesion >10 mm in diameter and 5 for gross coalescing lesions. Tissues were fixed in 10% formal saline and processed for histological examination following staining with haematoxylin and eosin (H&E). Tissues with typical lesions of TB evident by microscopy, but no gross lesions, were given a score of 1 in the overall pathology comparison.

Tissue samples were frozen at – 70°C for subsequent bacteriological examination by titration of tissue homogenates on modified 7H11 agar [26].

Antigens

Purified protein derivatives from *M. avium* (PPD-A) and *M. bovis* (PPD-B) were obtained from the tuberculin production unit at Veterinary Laboratories Agency (VLA), Weybridge, UK. Recombinant ESAT-6 and CFP-10 were as described [17].

Tuberculin skin tests

The single comparative intradermal tuberculin test with avian and mammalian PPD (PPD-A and PPD-B, respectively) was by intradermal inoculation of 0·1 ml of PPD-A and PPD-B and reactions read 72 h later. Results were recorded as increase in skin thickness at 72 h compared to thickness pre-injection and interpreted according to the standard protocol (European Communities Commission regulation number 1226/2002) [27].

Immunological assays

Blood was collected into heparin (10 U/ml). For cytokine assays 4 ml of blood was incubated at 37°C for 24 h with PPD-A, PPD-B (final concentration of 20 μg per ml) diluted in RPMI + 50 μg/ml gentamicin. ESAT-6 and CFP-10 were used at a final concentration of 5 μg/ml. An equal volume of RPMI + gentamicin was used as control. The supernatant was removed after centrifugation and stored at – 20°C until assayed. IFN-γ (pg) and IL-10 (units) were assayed by enzyme-linked immunosorbent assay (ELISA) as described using recombinant bovine standards [28]. Each sample assayed was measured in duplicate by ELISA; the variability between samples was less than 5%.

Secretion of IFN-γ by peripheral blood mononuclear cells (PBMC) was also assessed by enzyme-linked immunospot (ELISPOT) [29] with spots developed by the addition of 3-amino-9-ethylcarbazole (AEC) substrate (Kem-En-Tec Diagnostics) for 5 min, counted using the AID ELISPOT version 2.5 reader and software (Autoimmune Diagnostika, Strassberg, Germany) and are expressed as spot-forming cells (SFC) per 10^6 PBMC. The maximum number of SFC that can be read accurately is approximately 2000 per 10^6 PBMC.

An IL-4 ELISPOT assay was performed in a similar manner using MoAb CC313 and CC314-biotin (both produced at IAH) and shown to be effective in detecting natural IL-4 production in PBMC incubated with Con-A at 5 μg per ml (J. C. Hope, unpublished data).

Statistical analyses

Analyses were performed using MINITAB release 12·21. Differences in immunological responses, degree of pathology and bacterial colonization between BCG-vaccinated and control calves were compared using the Mann–Whitney *U*-test. Correlations between immune responses, bacterial burden and the degree of pathology were assessed non-parametrically using Spearman's rank correlation.

RESULTS

BCG vaccination of neonatal calves induces protection against disease caused by *M. bovis* challenge

One group of five 1-day-old gnotobiotic neonatal calves was vaccinated with BCG and another (control) group of five calves was inoculated with medium. Twelve weeks later both groups of calves were challenged intranasally with 10^4 *M. bovis*. The protective efficacy of BCG vaccination against disease was assessed by post-mortem examinations performed 16 weeks post-challenge (Table 1). None of the animals showed clinical signs of disease. However, lesions typical of TB were observed in four of five non-vaccinated control animals and in three of five BCG-vaccinated calves. In the BCG-vaccinated group the lesions were confined to head lymph nodes, whereas in four of five non-vaccinated calves lesions were evident in the lung or in the lung associated lymph nodes. The extent of lesions varied considerably between animals, reflected by the lesion scores (Table 1). However, both the number of tissues affected and the lesion scores of the BCG-vaccinated group were significantly lower than the control group, indicating that BCG had conferred a degree of protection against *M. bovis* challenge ($P < 0·05$).

BCG vaccination of neonatal calves reduces the level of bacterial colonization

Bacteriological examination of tissues indicated that *M. bovis* was present in numbers up to 5×10^4 CFU/g from the majority of tissues with gross lesions (20/30 tissues) but not from tissues with no lesions (3/90 tissues; Table 2), with a significant correlation between the number of isolated bacteria and lesion score ($P < 0{\cdot}01$). Fewer bacteria were present in tissues from the BCG-vaccinated group compared to the control group ($P < 0{\cdot}05$) and significantly fewer tissues contained bacteria ($P < 0{\cdot}05$).

Skin test reactivity in BCG-vaccinated and control calves following *M. bovis* challenge

Reactions to PPD in the skin test were observed in all animals 16 weeks after challenge with *M. bovis* (Table 3). Visible local clinical signs and increases in comparative skin thickness (PPD-B minus PPD-A) ranging from 3 to 31 mm were observed. Four of the five animals in each group would be classified as reactors, and one animal in each group inconclusive (animals no. 50 and no. 55), according to the standard interpretation of the tuberculin skin test. Using the severe interpretation of the test all 10 animals would be classified as *M. bovis* reactors. There appeared to be a trend indicating that BCG-vaccinated animals had lower skin test reactions compared to the non-vaccinated animals ($7{\cdot}2 \pm 2{\cdot}9$ compared to $13{\cdot}6 \pm 10{\cdot}2$), but these were not significant ($P = 0{\cdot}156$).

Immunological responses in BCG-vaccinated *versus* control cattle

Responses post-BCG vaccination

Immune responses were monitored for 12 weeks in calves vaccinated with BCG (Fig. 1a) and non-vaccinated animals (Fig. 1b). Significant increases in IFN-γ secretion in response to PPD were evident 2 weeks post-vaccination ($P < 0{\cdot}05$). The peak IFN-γ response to BCG was detected between 2 and 4 weeks post-vaccination and this had waned significantly by week 12. There was no significant correlation between the overall IFN-γ produced during the 12-week vaccination period and the extent of protection against disease as assessed by lesion score ($R^2 = 0{\cdot}046$). Although small responses to PPD were noted at some time-points in the control group (Fig. 1b), these were not increased significantly above the response seen with medium. No significant difference in IL-10 secretion was detected between BCG-vaccinated and control calves (data not shown).

Responses post-challenge

Twelve weeks post-BCG vaccination both groups of calves were challenged with *M. bovis*. Lymphocytes from both groups of calves secreted IFN-γ post-challenge (Fig. 1c,d). In the non-vaccinated group (Fig. 1d), IFN-γ secretion was observed by 3 weeks. At 4 weeks post-challenge there was significantly more IFN-γ secreted in response to PPD compared to the vaccinated group (Fig. 1c, $P < 0{\cdot}05$), suggesting a more rapid response to challenge in the control calves. There were consistently higher levels of IFN-γ secreted by calves in the non-vaccinated group (Fig. 1d) compared to the vaccinated group, but these differences were significant only at week 4. Each of the calves in the control group showed different reactivity to ESAT-6 and

CFP-10 (Fig. 2). Thus, calf no. 50 (Fig. 2a) did not respond to either antigen. ESAT-6 responses were observed in calves nos 54 (Fig. 2b), 57 (Fig. 2c) and 59 (Fig. 2e) but not in calf no. 58 (Fig. 2d), which responded only transiently to CFP-10. Responses in the vaccinated group to these antigens were absent or very low and intermittent (<200 pg/ml at any time).

Analysis of PPD-specific IFN-γ SFC by ELISPOT (Fig. 3) highlighted differences in the duration and magnitude of the IFN-γ response. There were more IFN-γ SFC in the non-vaccinated group than the BCG-vaccinated group and the responses were maintained for longer in the non-vaccinated calves. Thus, animals nos 54 (Fig. 3b), 57 (Fig. 3c) and 59 (Fig. 3e) showed high numbers of IFN-γ SFC throughout the 16-week challenge period, whereas responses in the BCG vaccinates nos 53 (Fig. 3g), 56 (Fig. 3i) and 60 (Fig. 3j) had waned significantly by 16 weeks. Interestingly, no. 50 (Fig. 3a), which had no visible lesions at post mortem, showed a positive ELISPOT response, suggesting a possible increased sensitivity of this assay for detection of infection.

IL-10 was produced in both the control and BCG-vaccinated groups; however, no significant differences were observed (data not shown). No IL-4 responses were detected using PPD as antigen.

Discussion

Studies of cattle vaccinated with BCG, as with humans, have shown variable efficacies. This variability has been attributed to sensitization by environmental mycobacteria [12,30,31]. Thus, the hypothesis that vaccination prior to exposure to

environmental mycobacteria might provide an enhanced level of protection obtained following BCG vaccination is worth thorough examination.

This study is the first to demonstrate the efficacy of BCG against *M. bovis* infection by the intranasal route. Vaccination significantly reduced the number of diseased tissues, the extent of tuberculous lesions and bacterial colonization. Bacterial spread to the lung and associated lymphoid tissue was limited in this model but was also reduced by vaccination. It is generally accepted that an effective vaccine should provide protection against disease, but would also have an efficacious effect by reducing or abrogating the transmission of infection. Our observations suggest that BCG vaccination of neonates may not only reduce disease, but may also have an impact on cattle-to-cattle transmission of infection.

The distribution of lesions induced by intratracheal/endobronchial instillation of *M. bovis* is similar to that observed in approximately 60% of animals that have been naturally infected with *M. bovis*. In these cases lesions are confined mainly to the lung and lymph nodes associated with the thoracic cavity [32–34]. Experimental infection with *M. bovis* via the intranasal route induces disease that involves the lymph nodes of the head and, to a lesser extent, those in the thoracic cavity and lung. This distribution of lesions is also evident in naturally infected cattle [32–35]. Recent studies employing an intratracheal/endobronchial challenge route have shown a significant level of protection of young cattle against *M. bovis* challenge. Vordermeier *et al.* demonstrated approximately 70% protection of 6-month-old calves following BCG vaccination [25]. Similar results were reported recently by Buddle *et al.* in calves vaccinated with BCG within 8 h of birth [15]. The intranasal challenge route has been used to produce disease in cattle and to produce cattle that transmit disease experimentally ([36] and unpublished). Vaccination linked to an

intranasal challenge route provides an alternative model for assessing vaccine efficacy and related immune responses. The spread of *M. bovis* between cattle is thought to be via the respiratory route through nasal secretions or the generation of aerosols within the upper respiratory tract [35], and is associated with the presence of lesions with lymph nodes of the head [36,37]. Thus, we suggest that a strategy to limit bacterial colonization in the head-associated lymph nodes could reduce the excretion of bacteria and consequent cattle to cattle transmission of disease.

Vaccination of 1-day-old calves induced strong IFN-γ responses to PPD-B evident within 1 week and peaking 2–4 weeks following vaccination, similar to other studies [15,20,38]. No correlation was found between the level of IFN-γ induced by BCG and the degree of protection; rather, high levels of IFN-γ are related to more extensive lesions [11,25]. Thus, the response to PPD-B was more rapid and significantly higher in the non-vaccinated group and was maintained at a high level for the entire post-challenge study period. Reduced bacterial replication in vaccinated calves is probably the explanation for the differences in kinetics and intensity of the IFN-γ response. There was a significant correlation between lesion score and PPD-B-induced IFN-γ, as reported previously [39]. In the vaccinated calves, the response of PBMC to PPD detected by IFN-γ ELISPOT had waned by week 16 post-challenge, whereas in the control calves the response was maintained at a much higher level throughout the study period. The number of PPD-B-specific IFN-γ SFC could be correlated with lesion scores (R^2 = 0·64). Thus, the two BCG-vaccinated animals with no visible lesions showed the lowest number of SFC. Conversely, the non-vaccinated and lesioned animals showed high numbers of IFN-γ SFC. The response pattern observed in vaccinated animals nos 53 and 56, where IFN-γ increases rapidly following challenge but is then reduced as the infection is controlled, is a useful correlate of

protection in experimental studies of bovine TB [38,40] and points to a way in which the level of response in ELISPOT assays in vaccinated animals may be used to distinguish vaccinated immune from vaccinated animals with disease.

What is required for cattle is a diagnostic test that not only identifies infected individuals and distinguishes them from vaccinates, but that also identifies individuals that are vaccinated but subsequently become infected and diseased. The ability to distinguish vaccinated and infected from vaccinated and immune animals needs to be on an individual animal basis, not just significant on a group or herd basis. This is necessary to identify accurately infected animals that are present in herds in low numbers and which may be a continuing source of infection. Differential diagnosis of BCG-vaccinated from *M. bovis*-infected cattle could be achieved using specific defined antigens that are present in virulent *M. bovis* but absent from BCG. These antigens include ESAT-6 and CFP-10 [41]. Approximately 70% of *M. bovis*-infected cattle were detected using a combination of ESAT-6 and CFP-10 [25,42], and reactivity to a cocktail of ESAT-6 and CFP-10 correlated with disease severity was useful for differential diagnosis of BCG vaccinated and *M. bovis*-infected cattle and could also distinguish between those cattle vaccinated with BCG that were protected from *M. bovis* challenge by the endobronchial route from those that were not [20,25,43]. The percentage of calves that reacted to ESAT-6 and/or CFP-10 in the current study is consistent with published observations, but it would appear that there are specific antigens other than ESAT-6 and CFP-10 present in PPD-B that remain to be identified and would be useful for diagnosis of infection. None of the BCG-vaccinated calves responded consistently to ESAT-6 or CFP-10 post-challenge. This includes both vaccinated and solidly immune calves and those that were vaccinated and diseased.

In summary, we have shown that vaccination of neonatal calves with BCG significantly reduces disease and bacterial load following intranasal challenge with *M. bovis*. Furthermore, the observations indicate that the specific antigens employed here will not necessarily distinguish BCG vaccinates with lesions from those without. However, it appears that a combination of as-yet-unidentified antigens and methods to assess the intensity and/or duration of the immune response can be developed and established as assays for this purpose.

Acknowledgments

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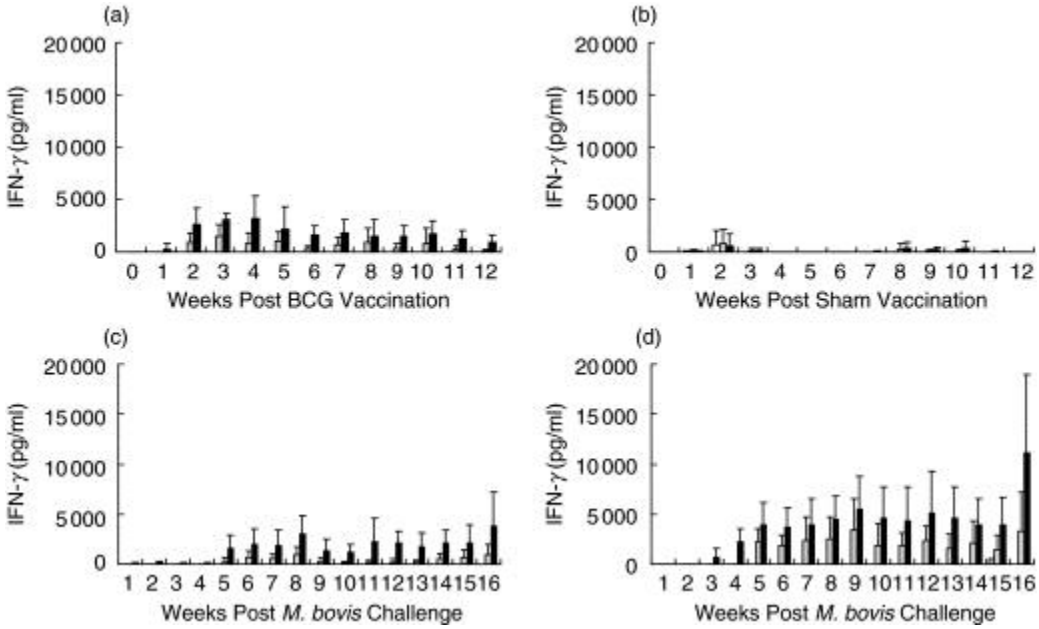
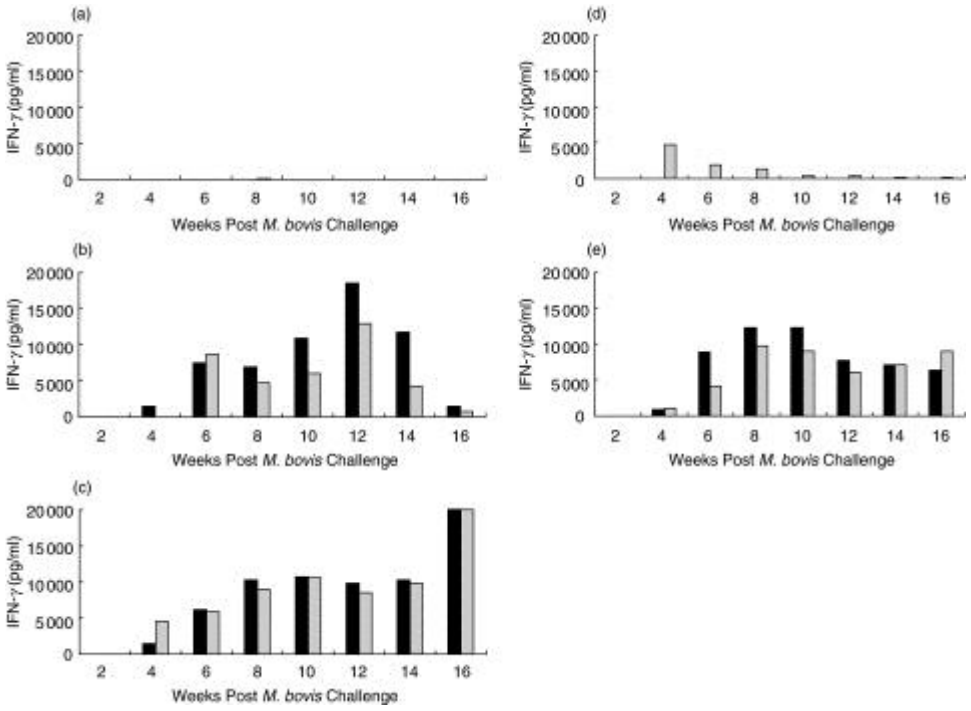
Figures

Fig.1



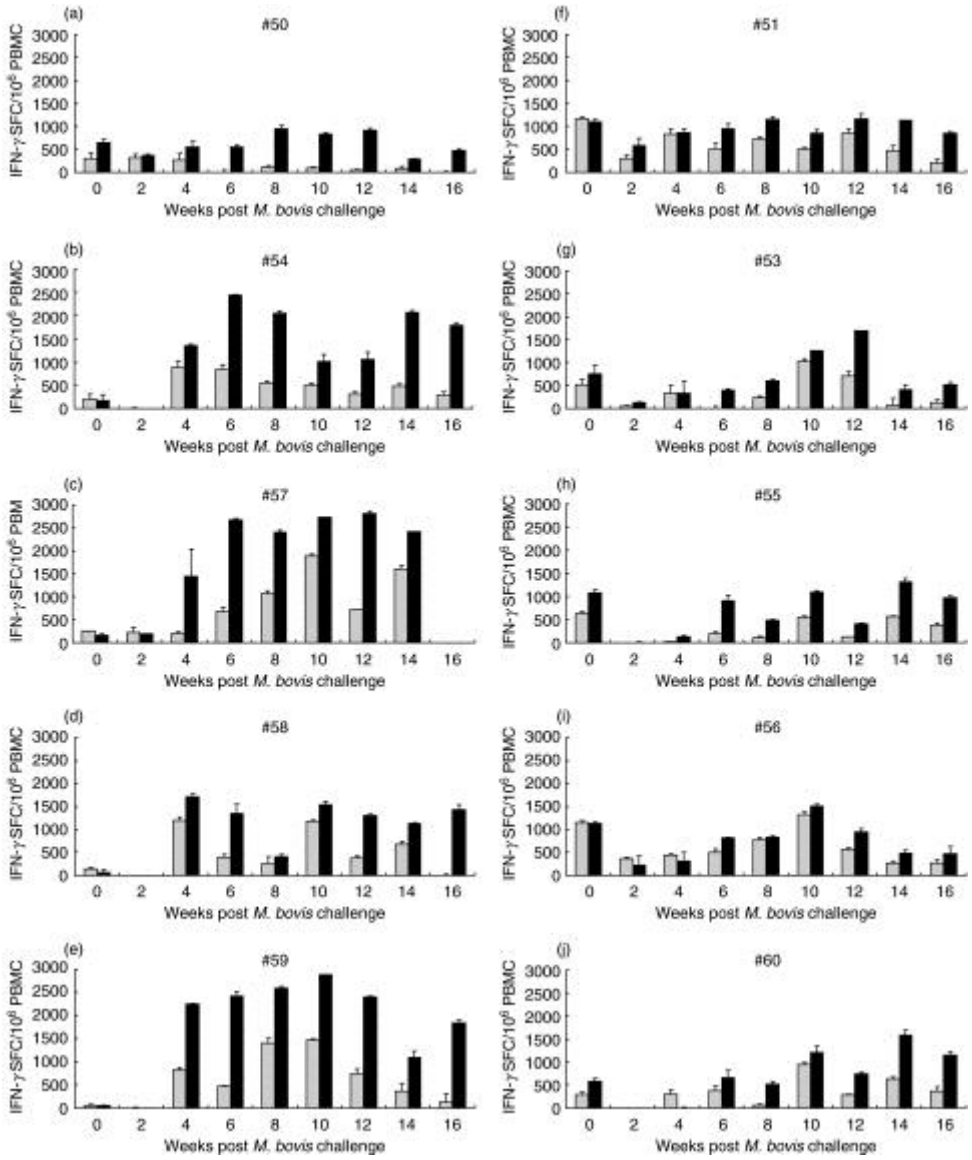
IFN-γ secretion post-BCG vaccination and post-M. bovis challenge. Gnotobiotic calves were vaccinated at 1 day (a) of age with BCG. Control calves (b) were inoculated with medium. Responses were measured for 12 weeks. Subsequently, both BCG-vaccinated calves (c) and control calves (d) were challenged intranasally with M. bovis. Whole blood was stimulated with PPD-A (grey), PPD-B (black) or medium control (white) and IFN-γ secretion in supernatants was assessed by ELISA. Means ± s.d. for groups of five calves are shown.

Fig.2



Responses of calves to *M. bovis* specific antigens. Five non-vaccinated calves (a–e) were challenged intranasally with *M. bovis*. Whole blood IFN-γ responses to ESAT-6 (black bars) and CFP-10 (grey bars) were assessed at the indicated times post-challenge. Results for individual animals are shown: (a) no. 50, (b) no. 54, (c) no. 57, (d) no. 58, (e) no. 59.

Fig.3



Detection of post-challenge IFN-γ responses by ELISPOT. PBMC from control (a–e) and BCG-vaccinated (f–j) calves were isolated at the indicated time-points post-*M. bovis* challenge. The number of PPD-A (grey bars)- and PPD-B (black bars)-specific IFN-γ SFC was estimated by ELISPOT. The number of SFC obtained by stimulation with medium control was subtracted from the number of SFC observed following antigen stimulation. Results for individual animals are shown.

Tables

Tab.1

Lesions at necropsy 16 weeks post *M. bovis* challenge in BCG-vaccinated and control calves. No lesions were evident in R and L tonsils, L and R diaphragmatic, L and R medial or intermediate lobes of the lung, or in three other bronchus associated lymph nodes.

	Animal no.									
	Controls					Vaccinates				
Tissue	50	54	57	58	59	51	53	55	56	60
Parotid R	0	0	3	1	4	0	0	0	0	1
Parotid L	0	0	3	0	4	0	0	0	0	1
Submandibular R	0	2	4	3	4	0	0	0	0	2
Submandibular L	0	2	0	0	4	0	0	0	0	0
Retropharyngeal R	0	0	4	4	4	0	0	2	0	0
Retropharyngeal L	0	4	4	4	4	2	0	2	0	0
Mediastinal	0	1	0	1	0	0	0	0	0	0
Bronchial 1	0	0	0	0	2	0	0	0	0	0
Nasal mucosa	0	0	0	0	2	0	0	0	0	0
Trachea	0	0	0	0	0	0	0	0	0	0
Apical R	0	0	2	0	2	0	0	0	0	0
Apical L	0	0	0	0	0	0	0	0	0	0
Total tissues affected	0	4	6	5	9	1	0	2	0	3
Total lesion score	0	9	20	13	30	2	0	4	0	4

L and R = left and right. Twenty-two tissues were examined for each animal. Score 0–4 indicates increasing severity of gross lesions.

Tab.2

M. bovis viable counts in tissues taken post mortem.

	Animal no.									
	Controls					Vaccinates				
Tissue	50	54	57	58	59	51	53	55	56	60
Parotid R	0	0	3.19[a]	3.38	4.54	0	0	0	0	0
Parotid L	0	0	0	0	4.27	0	0	0	0	0
Submandibular R	0	0	3.56	3.73	4.21	0	0	0	0	3.24
Submandibular L	0	3.27	0	2.74	4.35	0	0	0	0	2.78
Retropharyngeal R	0	0	3.85	4.03	4.19	0	0	3.28	0	2.85
Retropharyngeal L	0	3.94	3.76	3.90	4.51	3.73	0	3.67	3.44	0
Tonsil R	0	0	0	0	0	0	0	0	0	0
Tonsil L	0	0	0	0	0	0	0	0	0	0
Total no. of tissues affected	0	2	4	5	6	1	0	2	1	3

aViable count (log10 CFU per g tissue). The limit of detection using the isolation technique is five colonies. No isolations were made from nasal mucosa, trachea, R and L apical lobes of lung apart from nasal mucosa of no. 59 (3.51 CFU per g). Twelve tissues examined for each animal.

Tab.3

Skin test responses 16 weeks post-challenge with M. bovis.

	Animal no.									
	Controls					Vaccinates				
Tissue	50	54	57	58	59	51	53	55	56	60
PPD-A	1[a]	4	4	5	4	4	0	4	7	7
PPD-B	4	14	35	16	16	13	7	8	12	18
(PPD-B)-(PPD-A)	3	10	31	11	12	9	7	4	5	11

a Increase in skin thickness from day 0 to day 3.

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